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(54) Title: MONOCLONAL ANTIBODIES TO THYMIDINE KINASE ISOZYMES

(57) Abstract

A monoclonal antibody which selectively binds to all forms of thymidine kinase, including TK2, active and inactive forms of TK1, and monomeric and multimeric forms of TK1, is described. Also described are monoclonal antibodies which selectively bind only to active or tetrameric TK1 and to TK2, respectively. A panel of antibodies including two or more of the above-described antibodies is useful to correlate levels of TK1 separately from total TK or TK2, according to a method described in the application. Methods for producing the above-noted monoclonal antibodies are also described, including a method for obtaining sufficient quantities of pure active/multimeric TK1 for use as an antigen. The anti-TK antibodies in the panel are useful individually and in various combinations to test tissue samples of human tumor patients, to predict from a primary tumor the likelihood of tumor recurrence, and to monitor treatment effectiveness. The antibodies are also useful for diagnosing, staging, and monitoring treatment efficacy in tumors of other kinds.

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MONOCLONAL ANTIBODIES TO THYMIDINE KINASE ISOZYMES

BACKGROUND OF THE INVENTION

Field: The invention relates generally to a monoclonal antibody useful for diagnosis, prognosis, and treatment assessment of cancer and certain blood disorders, and to prediction of recurrence in cancer patients.

State of the Art: Once a cancer is detected, an important factor in determining what treatment(s) to use is the stage of development of the disease. Generally, cancers are graded from Stage I, the earliest and most treatable phase, to Stage IV which is a very advanced, often metastatic tumor with prognosis of death.

Also, it is desirable to monitor the growth or lack thereof, of the cancer during and subsequent to a course of treatment. If tumor recurrences are detected very early, they may in some cases be successfully treated to produce an ultimate cure or to significantly prolong the life of the patient. Or, if a tumor is not responding well to a particular drug or treatment, others can be tried.

Taking breast cancer as an example, if it were possible to accurately predict those patients that were liable to exhibit tumor recurrence, then appropriate courses of treatments could be administered dependent on the aggressiveness of the tumor. In cases where the tumor is believed to be very aggressive, then close follow up is called for with additional surgery, radio-therapy, and possibly chemotherapy. If the tumor is shown not to be aggressive, then a milder course of treatment may be appropriate. Accurate prediction of breast tumor behavior would clearly facilitate better tumor management and contribute significantly to patient well being.

Also, early detection of recurrence would enable the physician to take rapid measures to treat the recurrence, and possibly increase the likelihood of patient survival.

For breast cancer, it is presently believed that one of the best predictors of the likelihood of recurrence is estrogen receptor status. Those patients that are estrogen receptor positive (high numbers of estrogen receptors on the tumor cells) are thought to have a better prognosis, while those that are estrogen receptor negative are thought to have a poor prognosis. However, there are many cases where those who are estrogen receptor negative do not show recurrence, and those that are estrogen receptor positive do show recurrence. Because the estrogen

receptor test is not sufficiently reliable as a prognostic indicator, patients are often treated with both radiotherapy and chemotherapy, regardless of estrogen receptor status.

Thymidine kinase (ATP:thymidine-5' phosphotransferase; EC 2.7.1.21 in the International Union of Biochemistry classification system) is an enzyme that phosphorylates thymidine to thymidine monophosphate (TMP). The commonly used abbreviation of TK will be used herein to denote thymidine kinase in a general sense, including different isozymes and multimeric forms presently believed to exist **in vivo**.

Thymidine kinase protein has been isolated from many different sources and purified to varying degrees. A variety of different molecular weight thymidine kinases have been reported from human samples, depending on the particular cell and the method of isolation and analysis (for example, under denaturing conditions vs. non-denaturing conditions). In general, the findings suggest that thymidine kinase exists in at least one monomeric form of MW 24-28 KD, and a variety of multimeric forms.

In humans, it is also known that there are at least two major isozymes (similar but distinct forms) of thymidine kinase, referred to herein as TK1 and TK2. These isozymes are produced from different genes, are found in different cellular compartments, and differ in their levels and timing of expression during the cell cycle and according to the cell differentiation state. In humans, the TK1 gene is on chromosome 17 in band q21-22 (Elsevier 1974) while the TK2 gene is on chromosome 16 (Willecke 1977). A gene for TK1 has recently been cloned and sequenced (Lin 1983, Flemington 1987).

There are reports that TK activity is elevated in the serum or tumor tissues of patients with some kinds of cancer, including acute and chronic leukemias, Hodgkins' and non-Hodgkins' lymphomas, and solid tumors of breast, prostate, brain, and rectum. Persistent elevation of thymidine kinase in the serum has been proposed as an indicator of malignant disease. However, the measurement of thymidine kinase activity by conventional means is tedious and not always reproducible.

Furthermore, it is not always clear from previous reports which of the isozymes or forms of thymidine kinase is elevated in activity in cancer patients. The method generally used for measuring the level of TK1 is based on enzyme activity by comparison of incorporation of radioactive thymidine in parallel samples assayed with different secondary substrates (adenosine triphosphate or ATP, and cytosine triphosphate or CTP). Both TK1 and TK2 utilize ATP as the substrate very efficiently. However, the TK1 isozyme has only about 7-15% activity with CTP as substrate, as it does with ATP. TK2, however, is nearly as efficient with CTP as with ATP. The levels of total TK activity are determined from the assay with ATP, while the levels of TK2 activity are determined from the assay with CTP. The difference between the activity level measured with CTP and that measured with ATP is attributed to the TK1 isozyme.

This method is tedious, and the results are highly dependent on the precise performance of the parallel assays and sometimes difficult to reproduce. Further, since TK1 does show some incorporation with the CTP substrate, the interpretation can be ambiguous. Also, the active form of the TK1 protein appears to be rather unstable, so the amount of activity detected may vary depending on handling of the sample.

It would thus be desirable to be able to measure the amounts of TK protein and/or of individual TK isozymes. For such purposes, an antibody specific for a TK isozyme, especially a monoclonal antibody, would be useful.

European Patent Publication No. 0 042 482 of Balis et al., published December 30, 1981, discloses a process for detecting malignant and pre-malignant cells in humans using an antiserum raised against an isozyme of thymidine kinase termed "oncofoetal" TK. The TK isozyme used to obtain the antiserum was isolated from human term placental material. However, the Balis antibody did not react with leukemic leucocytes or with normal or mitogen-stimulated peripheral lymphocytes, even though these are known to have elevated TK levels (Balis et al., col. 2, lines 21-23).

Another European Patent publication, No. 0 255 431 by Jouan published October 23, 1991, discloses purification of "TK-F", that is, fetal TK, from human

placental material for purposes including use of the pure TK-F to produce anti-TK-F antibodies. However, they do not disclose actually obtaining such an antibody.

Accordingly, a need remains for antibodies useful to detect the total amounts of thymidine kinase enzymes serum and tissues. A need also remains for a panel of antibodies useful to distinguish different TK isozymes and active vs. inactive forms of TK. There further remains a need for improved methods to assess treatment efficiency and likelihood of tumor recurrence in breast cancer patients and in patients with other types of tumors.

Summary of the Invention

The invention comprises a series of monoclonal antibodies which are specific for various isozymes of thymidine kinase. Among the monoclonal antibodies encompassed in the series are a monoclonal antibody which specifically binds to "total TK", defined as including both TK1 and TK2. Another anti-thymidine kinase antibody is specific for protein having 90% or greater sequence homology to TK1. Another antibody of the series specifically binds to "active TK1", defined as a multimer form of TK1 which has activity to phosphorylate thymidine. Still another antibody of the series specifically binds to "total TK1", defined as comprising active TK1, inactive TK1, monomeric TK1, and multimeric TK1. Some embodiments of the anti-active TK1 and anti-total TK1 antibodies also are configured to inhibit the phosphorylation activity of TK1 upon specific binding thereto. The invention further embraces monoclonal antibodies which selectively bind only to tetrameric TK1, and only to TK2, respectively.

In another embodiment, the invention comprises a panel or kit containing two or more monoclonal antibodies useful to determine the relative amounts of TK1 vs. TK2 and/or the relative amounts of active and inactive TK1. A preferred embodiment of such a panel includes a first antibody specifically binding to TK1 and a second antibody which specifically binds "total TK" or, both TK1 and TK2. Still another embodiment comprises a panel of monoclonal antibodies useful to evaluate the relative amounts of active TK1 vs. total TK1. Such a panel may include an antibody specifically binding total TK1 and an antibody specific for active TK1.

The invention further embraces hybridomas producing the noted monoclonal antibodies, methods for obtaining such hybridomas, and methods of using the monoclonal antibodies in a clinical setting with human patients, to predict the likelihood of recurrence of a solid tumor and to monitor treatment effectiveness. 16.

A method of making a hybridoma cell line which produces a monoclonal antibody specific for thymidine kinase comprises the steps of providing a thymidine kinase preparation; injecting a host animal with the thymidine kinase preparation, and waiting a sufficient time for the animal to mount an immune response against the antigen preparation; removing activated B lymphocytes from the injected animal, fusing the activated B lymphocytes with myeloma cells to form a plurality of hybridoma cells and individually culturing the hybridoma cells to produce a plurality of hybidoma clones; and screening the hybridoma cultures to select a hybridoma clone which secretes an antibody that binds with specificity to a thymidine kinase isoenzyme. In one embodiment, the thymidine kinase preparation is a substantially homogeneous preparation of active TK1 or multimer TK. In another embodiment, the thymidine kinase preparation is derived from Raji cells, which as taught in the application are a source of pure TK1. A screening procedure for selecting monoclonal antibodies with desired specificities utilizes 1) a crude TK1 preparation containing monomer, multimer and active and inactive forms of TK1 (which may be obtained from Raji cells); 2) a crude TK2 preparation (possibly obtained from HeLa cells); and 3) active TK1 purified to a form which when subjected to non-denaturing electrophoresis, migrates as a homogeneous multimer species. The screening procedure may also include screening to determine the ability of antibodies to inhibit thymidine kinase activity in cell extracts, and/or Western blotting of electrophoresed preparations of thymidine kinase.

A method of predicting the likelihood of recurrence of a tumor in a patient at initial diagnosis comprises the steps of establishing a normal range for tissue TK activity, obtaining a sample of a primary tumor from a patient, determining the amount of TK enzyme in the patient sample to produce a patient TK value, and comparing the patient TK value it to the normal value; and if it exceeds the normal range by a significant amount, predicting that the tumor is likely to recur, and if it

does not significantly exceed the normal range, predicting that recurrence is unlikely.

The invention also provides improved methods and compositions for diagnosing and staging solid and leukemic and lymphoid tumors, and for monitoring treatment efficacy and detecting recurrence of such tumors. A method of determining whether disease has recurred in a patient being treated for a leukemia or lymphoid cancer comprises the steps of taking a series of samples of the serum of a cancer patient at regular intervals, measuring the amount of TK in the samples, comparing the amount of TK among the samples, and when the amount of TK in later samples exceeds the amount of earlier samples by a significant degree, determining that the disease is recurring.

Brief Description of the Figures

FIG. 1 is a chart depicting absorbance at 280 nm plotted versus elution time from a DEAE-cellulose column of a crude extract of TK1 prepared from Raji cells according to the invention;

FIG. 2 is a chart depicting absorbance at 280 nm plotted versus elution time from a Mono Q column of a crude TK1 preparation run as described in Example IV;

FIG. 3 is a chart depicting absorbance at 280 nm plotted versus elution time from a Mono Q column of a partially purified TK1 preparation corresponding approximately to fractions 201-204 of FIG. 2, subjected to a second run on a Mono Q column as described in Example IV;

FIG. 4 is a chart depicting absorbance at 280 nm plotted versus elution time from a Mono Q column of a further purified TK1 preparation corresponding approximately to fractions 308, 310 of FIG. 3, and subjected to a third run on a Mono Q column as described in Example IV;

FIG. 5 is a chart depicting absorbance at 280 nm plotted versus elution time from a Mono Q column of a crude extract of TK prepared from HeLa cells and run as described in Example IX;

FIG. 6 is a chart depicting absorbance at 280 nm plotted versus elution time from a Mono Q column of a partially purified TK2 preparation corresponding

approximately to fractions 501-504 of FIG. 5, subjected to a second run on a Mono Q column as described in Example IX;

Detailed Description of the Preferred Embodiments

In the development of an antibody specific for TK1, and especially for an anti-active TK1 antibody, several difficulties were encountered. The active form of TK1 is rather labile, so it is difficult to prepare it in sufficient purity and amount for use as an antigen. Because of its lability, the active multimer form of TK1 may be degraded to monomers or inactive forms after injection into the mouse and before B-cells making antibodies specific to the active or high MW TK1 are formed, so that the probability of obtaining a hybridoma producing such antibodies is severely reduced. Further, mice have a TK1 enzyme, so it is difficult to elicit an antibody-forming response to TK1 protein.

As part of the invention, it was discovered that Raji cells appear to produce only a single TK isozyme, believed to be TK1. Raji cells are an immortalized human lymphoma cell line, available from ATCC as cell line #CCL-86 The discovery of this pure antigen preparation was important in overcoming the obstacles which have interfered with previous attempts to produce anti-TK1 antibodies.

EXAMPLE I

Assay of Raji cells for TK1 and TK2 activity. A crude cell extract was prepared from Raji cells as follows. Approximately 10^{11} to 10^{12} exponentially-growing Raji cells were harvested by centrifugation from the growth medium. The pelleted cells were separated from the supernatant and resuspended in 1-2 mls of extraction buffer containing 0.02 M Tris-HCl, pH 7.8, 0.05 M $MgCl_2$, and 0.2 mM KCl. The cell suspension was subjected to three freeze-thaw cycles in liquid nitrogen and a 37°C waterbath. The ruptured cell suspension was then centrifuged at 30,000xg for 30 minutes to pellet cellular debris. The supernatant, containing about 50 mg/ml of protein, including TK and other soluble enzymes, was decanted from the pellet and stored frozen at -20° C.

To perform TK assays, 0.2 milliliter (abbreviated ml) of the crude extract was mixed with an equal amount (0.2 ml) of an assay mixture containing 0.02 M

Tris-HCl (pH 7.8), 2 x 10-6 M [^{3}H]-thymidine (85 curies per millimole), 0.002 M MgCl2, 0.2 M KCl, 0.1 M NH4Cl, 0.005 M mercaptoethanol, and 0.004 M ATP (adenosine triphosphate).

The assay reactions were incubated at 37° C in a water bath. After 30 minute and 60 minute incubation periods, 0.025 ml samples were removed and spotted onto Whatman DE-81 discs. The filter discs were allowed to dry and washed 3 times with 0.01 M formate for 5 minutes each time. The discs were then rinsed with distilled water for 5 minutes followed by rinsing with methanol, and transferred to scintillation vials containing 4 mls of scintillation counting fluid for measurement of ^{3}H radioactivity. A duplicate assay was performed in the same manner but substituting CTP (cytosine triphosphate) for ATP.

The results indicated that the crude extract of Raji cells incorporated 7000-8000 cpm of CTP in 60 minutes, as compared to about 259,000 cpm incorporated when ATP was used as the precursor. These results were considered to indicate that there was no detectable level of TK2 activity in Raji cells. The specific activity of the Raji extract was about 559 cpm/mg protein/min.

EXAMPLE II

Partial purification of TK1. TK1 enzyme was partially purified from the crude extract of Raji cells of Example I by DEAE-cellulose anion exchange chromatography. To obtain the largest yields of TK protein, it is desirable that the cells be in the exponential growth phase when harvested. The protein content of the crude extract was determined using the well known Bradford assay. A total of about 1.0-2.0 grams of protein from the crude extract was added to a DEAE-cellulose column and washed with 10 void volumes of 0.1 M Tris-HCl (pH 8.0) using gravimetric flow. The column was eluted with 0.5 M Tris-HCl (pH 8.0), and 1.0 ml fractions were collected.

FIG. 1 depicts the absorbance measured at 280 nm as a function of elution time. The collection of one-ml fractions 102, 103, 104 is indicated. Portions of the collected fractions were assayed for TK1 activity generally as described in Example I. Fraction 104 approximately spans a first peak 110 in the chromatograph, which was found to contain most of the TK1 activity eluted from the column. By pooling and concentrating fraction 104 from about a hundred runs performed as in Example

II, approximately 40 mls of eluant containing about 1.6 mg/ml of TK1 protein were recovered. The approximate specific activity of the pooled DEAE-cellulose preparation was 17,430 cpm per mg protein per minute.

The pooled DEAE-cellulose fractions were concentrated using an Amicon protein concentrator, and a sample was electrophoresed under non-denaturing conditions on a 10% polyacrylamide separation gel with a 4.0% polyacrylamide stacking gel. Approximately 7 bands were visible in the gel, ranging from about 24,000 to about 180,000 daltons in molecular weight (MW). These bands were cut out, the protein eluted from the gel and assayed for TK1 activity in a manner similar to that described in Example I. Only one band of about 100,000 daltons contained significant TK1 activity. There was no significant TK1 activity in any of the other bands. The 100,000 MW band comprised a semi-pure preparation of active TK1, and was used as the antigen to produce anti-TK1 monoclonal antibodies. About 50 μg (micrograms) of this semi-pure TK1 was recovered from the pooled DEAE-cellulose prep.

Example III

Purification of TK1 by ROTIFER. Alternatively, TK1 was partially purified by isoelectric focussing of the curde extract using a ROTIFER apparatus purchased from Bio-Rad. The procedure used was that outlined in the ROTIFER manual from Bio-Rad (1990). Six to seven protein bands were observed in the isoelectric gels, one of which had a molecular weight of about 100,000 daltons and exhibited some TK1 activity when assayed as described in the preceding paragraphs. The recovery of activity was rather poor, compared to the methods of Examples II and IV.

Example IV

Purification by FPLC. A third and presently preferred method of purification of TK1 employs FPLC (Fine Protein Liquid Chromatography) with three sequential purifications on a Mono Q 5/5 anion exchange column, using different elution gradients for each run. The Mono Q 5/5 is an ion exchange column packing commercially available from Pharmacia, having substantially monodisperse bead size and strong anion exchange properties due to bound quaternary amine groups which remain charged over the range from pH 2 to pH 12.

Apparatus for the procedure can also be purchased from Pharmacia. The column was loaded with 0.1 ml of the semi-pure preparation from the procedure of Example II, containing about 1 mg protein, and voided with 10 volumes of Buffer A (50 mM Tris-HCl, pH 8.0). The void volume of this column was 1.0 ml. A programmed gradient was set up to gradually increase the concentration of Buffer B (1.0 M NaCl, 50 mM Tris-HCl, pH 8.0) from 0-100% over 20 minutes running at a constant flow rate of 1.0 ml/min.

The protein was detected as it eluted from the column by absorbance at 280 nm (FIG. 2). Fractions containing the 280 nm absorbance peaks were collected and assayed for TK1 activity as described previously herein. TK1 activity was determined to be primarily in peak 200 (the first peak eluting from the column), at which point the gradient contained about 15-20% of Buffer B.

Protein from the peak 200 was analyzed by non-denaturing PAGE as before to determine purity. There were 5 protein bands present. These bands were cut out and protein from each was assayed for TK1 activity. Detectable TK1 activity was found in the high molecular weight band (100,000 MW), but not in the other bands.

For a second purification step, the TK1-activity-positive fractions from several runs were collected, pooled and concentrated. This partially purified, pooled sample was then re-run on the Mono Q column with a lower gradient. One-tenth ml portion of pooled sample containing about 1 mg protein, was loaded on the Mono Q column as before. For this second run, the gradient was started at 5% of Buffer B and ran to 40% Buffer B over 35 minutes at 1.0 ml/min.

FIG. 3 depicts a chromatogram of absorbance vs. elution volume for the second sequential Mono Q run. Fractions containing 1.0 ml of eluant were again collected. A major peak 300 eluted from the column at about 15% of Buffer B, and two minor peaks 302, 304 eluted at about 18% and 20% Buffer B respectively. The peaks were assayed for TK1 activity and fraction 310 from the major peak 300 was determined to contain TK1 isozyme activity.

Protein from peak 300 (fractions 308, 310) was also analyzed by SDS-PAGE, and found to contain 3 proteins of molecular weights of about 100,000, 75,000, and 24,000, respectively. Upon assay for TK1 activity of protein from

each of the bands, only the 100,000 dalton protein exhibited significant TK1 activity.

A third sequential Mono Q run was performed on protein precipitated and pooled from fractions 308, 310 containing the peak 300. The running conditions were further changed by slowing the flow rate and further decreasing the gradient. A gradient of 5% Buffer B to 30% Buffer B was run at 0.5 mls/min for 50 minutes. For this run, 0.5 ml fractions were collected. There were three peaks 400, 402, 404 eluting near 15% of Buffer B. Protein from each of peaks 400, 402, 404 was assayed for TK1 activity as before. The first peak 400 to elute contained TK1 activity, and when analyzed by non-denaturing PAGE was found to contain a single band at a molecular weight of about 100,000. This preparation is designated herein as "purified" TK1 isozyme. About 5 nanograms of protein with a specific activity of about 2,412,800 cpm/mg protein/min, were recovered in peak 400.

The protein from peak 400 from several column runs was pooled and analyzed by electrophoresis under reducing and non-reducing conditions. Only one band at 100,000 MW was observed on the non-reducing gel, while a faint band at 100,000 MW and a darker band at 24,000 MW were observed on the reducing gel. From this result it appears that the active form of TK1 may be a multimer with subunits of lower molecular weights. The subunits also appear at present, to be of identical molecular weights.

Presently, the procedure of Example IV using the FPLC with Mono Q column, is the preferred method for isolating pure TK1. In Example IV, the starting material was the crude extract of Example I. However, alternatively the DEAE-cellulose preparation of Example II can be used as the starting material. For producing semi-pure TK1 for antigen injection into mice, however, the method of Example II may be used, or the product obtained by the second run on the Mono Q column as in Example IV, is also suitable.

EXAMPLE V

Production of monoclonal antibodies binding to TK1. Hybridoma cell lines producing antibodies to TK1 were produced by methods generally known in the art, but with certain modifications.

The immunization technique is different than those found in the literature. Semi-pure TK1 was prepared as in Example II. A dose of 100 μg of semi-pure TK1 suspended in 50 μl of PBS + 50 μl complete Freund's adjuvant was given intraperitoneally (I.P.) to each of a group of female BALB/c mice, 5-6 weeks old. Two weeks later, a second immunization was given that was identical to the first.

Two weeks following the second immunization with semi-pure TK1, an intrasplenic injection was given which contained 10 μg of pure active TK1 (prepared as in Example IV) suspended in 100 μl of PBS. The mice were anesthetized with Sodium Pentobarbitol (65 mg/ml) which was diluted by adding 6.7 mls to 93.3 mls of PBS. Each mouse was given 10 μl/gram of body weight I.P. Surgical intervention was performed using a scalpel and forceps, and the spleen was gently teased out for administration of the antigen. Several areas of the spleen were injected to insure uniform distribution of the antigen. The wound was closed with metal sutures and the mice were placed under a heating lamp for 1-2 hours.

Seventy-two hours following the intrasplenic injection, the mice were sacrificed using ether and the spleen was removed. Before the mice were killed, blood was removed and the serum tested to insure that the mice were mounting an immune response to the TK1 protein. The B cells were isolated from the spleen for fusion with an immortal myeloma cell line.

The cell line used for the fusion partner was a self-fused Sp2/0 line designated FO which was purchased from ATCC. It is a derivative of P3-X63-Ag8. This line is an immortal myeloma mouse cell line that is fast growing and a non-secretor (heavy or light chain immunoglobulins). The fusion of FO and activated spleen cells was performed generally as known in the art. One spleen containing about 1 x 10^8 cells was used per fusion. The most successful fusions resulted when the ratio of B-cells to FO cells was about 10:1. After the fusion was terminated, the fusion cell suspension was seeded into 96-well microtiter plates which had been seeded a day earlier with 3,000 to 6,000 mouse macrophages per well as feeder cells.

HAT selection medium was used to select only fusion products. Wells were marked for growth and gradually weaned out of HAT and into regular media. By this time the only surviving cells were hybridomas obtained by fusion of B-cells and

FO cells. A total of about 500 colonies representing fusion products, resulted from each fusion.

EXAMPLE VI

Preliminary screening of hybridoma colonies from fusion. 500 colonies from one fusion were subjected to preliminary screening by ELISA against partially purified TK1 prepared as in Example II. Supernatants collected from the hybridoma cultures were initially screened with semi-pure TK1 prepared by running the crude extract of Raji cells on DEAE cellulose to partially purify the TK1. Thus, this preliminary screen detects antibodies to multiple forms of TK1, including monomer, multimer including tetramer, active and inactive forms, etc.

Multiwell plates were coated with 1.0 μg (micrograms) per well of selected TK protein preparations suspended in 50 μl PBS, and allowed to dry overnight. The plates were then treated for 30 minutes with 200 μl per well of PBS-Tween 20-EDTA-1% milk fat, to block non-specific binding. The plates were washed 3 times with 200 μl of PBS-Tween 20-EDTA (PBST2E). Tween 20 is an anionic detergent commercially available from Bio-Rad Laboratories, Richmond, CA, and useful to reduce non-specific antibody-antigen binding while not disrupting binding of primary antibodies to antigens or of antigens to nitrocellulose.

The growth medium on the hybridoma cell cultures was not changed for three days prior to collection of the hybridoma culture supernatants, in order to saturate the media with antibodies. For each hybridoma, 80 μl of supernatant per well was added to duplicate wells. The multiwell plates were then incubated at 37° C for 1-1/2 hours. The supernatant was decanted and the wells washed 6 times with PBST2E.

Next, goat anti-mouse IgG (heavy and light chain specific) conjugated with peroxidase (available from Bio-Rad) and diluted 1:3,000 in PBST2E was added. One-tenth ml was added to each well and the plates incubated as before. The wells were again washed in PBST2E and 200 μl of substrate, tetramethyl-benzidine, was added and incubated for 1 hour. The substrate reaction was stopped by adding 50 μl of 2 M sulfuric acid to each well to cause a color shift from to blue to yellow. The plates were scanned for O.D. measurement at 450 nm on a plate reader. O.D. readings that were at least twice the background O.D were deemed positive. Of

about 25,000 clones obtained from fifty fusions, thirty-five tested positive in the preliminary screening. The positive colonies were isotyped using a kit from Hyclone, Logan, UT (cat. # EK-5051), and the positive colonies determined to produce antibodies of IgG1, IgG2a, IgG3, and IgM classes.

EXAMPLE VII

The 35 clones which tested positive in the initial screenings were subjected to more rigorous screening. A plate was coated with five pairs of replicate wells as follows: wells A,B were coated with a crude extract of TK1 from Raji cells; wells C,D were coated with semi-purified TK1 prepared from the DEAE-cellulose column; wells E,F were coated with purified TK1 from peak 400 prepared as in Example IV by FPLC (see FIG. 4); wells G,H were coated with TK1 protein from fractions 308,310 of the second FPLC run (see FIG. 3); and wells J,K were coated with an extract of **E. coli** cells which expressed a TK1 gene in a PET vector. For the purified samples, samples, 1.0 μg per well of protein was used.

The ELISA was performed essentially as described for the preliminary screening. Of the 35 clones tested, one proved to bind only to active form TK1. The absorbance readings (ABS) were made at 405 nm for 120 wells on one plate on which ten clones were screened are shown in TABLE I. The clones testing most highly positive by preliminary screening were purposely clustered on this plate. The background ABS from four wells was averaged and found to be about 0.058 (wells J11, J12 and K11, K12).

It is apparent that positive binding (absorbance significantly greater than the background level) was observed in all the wells in columns 2 and 5; in all but rows C,D of col. 1; in rows J,K of columns 3, 4 and 7; and in rows A,B of columns 4 and 7. That is, the clones in columns 1-5 and 7, all tested positive for binding to TK1. Of these, clones nos. 2 and 5 tested positive for binding to all of the TK1 preparations, while clone no. 1 bound to all the TK1 preparations except the semi-purified DEAE-cellulose preparation. Clones nos. 4 and 7 bound to the crude Raji cell TK1 extract and to the TK1 produced by genetic engineering in **E. coli**. Clone no. 3 bound only to the TK1 produced from **E. coli**. The remaining 25 clones tested negative for antibodies to TK1.

Table I

Screening of Hybridoma Clones for Anti-TK1 Antibodies

TK PREP.	CLONE NUMBER											
	1	2	3	4	5	6	7	8	BLANK	BLANK	11	12
A	.196	.204	.137	.227	.165	.055	.090	.067	.075	.057	--	--
B	.403	.181	.117	.172	.153	.049	.091	.068	.086	.050	--	--
C	.061	.109	.050	.156	.119	.041	.048	.049	.045	.039	--	.000
D	.078	.117	.063	.090	.158	.046	.049	.046	.046	.046	--	--
E	.114	.150	.085	.116	.157	.043	.046	.114	.083	.056	--	--
F	.160	.142	.083	.127	.218	.050	.114	.076	.081	.045	--	--
G	.093	.128	.081	.135	.164	.057	.076	.071	.086	.048	--	--
H	.107	.141	.097	.153	.222	.056	.071	.059	.064	.051	--	--
J	.240	.291	.188	.195	.225	.063	.116	.068	.077	.126	.054	.071
K	.243	.197	.195	.182	.215	.077	.124	.073	.074	.069	.058	.082

Values given are the O.D. measured at 405 nanometers (not 450 nm) for samples assayed by ELISA

Further characterization was performed by Western blotting. The Western blots were prepared by procedures similar to those described in CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, publ. Wiley-Interscience, New York (1991). Antibodies were harvested from the supernatant of each hybridoma and hybridized to a nitrocellulose membrane blotted from a non-denaturing gel of TK proteins. A goat anti-mouse IgG was then used for detection of the bound antibodies.

In a blot obtained by electrophoresis of a sample of Raji extract, it was observed that the Clone #1 antibody bound only to the 100,000 dalton band known to have activity. Clone 5 antibody also bound only to this band, but less strongly. Clone 2 antibody bound to four bands of molecular weights about 24,000, 48,000, 72,000 and 100,000. These are believed to represent monomer, dimer, trimer and

multimer forms of TK1. Thus, the Clone 2 antibody binds to all the commonly observed forms of TK1, or "total" TK1.

Another Western blot was prepared from a gel in which the purified multimer TK1 from the third run of the FPLC procedure (peak 400 in FIG. 4). This purified multimer migrates as a homogeneous species at a molecular weight of 100,000, but has considerably less activity than was expected. Thus, this preparation is a multimer or tetramer form, but may not be in the active configuration. In this blot, the Clone 5 antibody bound strongly to the one band at 100,000, while the Clone 1 antibody bound less strongly to the band - the opposite of the result obtained in the blot of Raji cell extract.

From these results it can be seen Clone 1 and Clone 5 bind to different epitopes. The Clone 1 antibody binds selectively to the active multimer form of TK1, and is designated hereinafter as an "anti-AcTK1" antibody. Clone 5 antibody binds to the multimer form, but apparently preferentially to an inactive multimer form.

Clones Nos. 1-5 are all IgM-type hybridomas. Clone 7 is an IgG-type hybridoma.

EXAMPLE VIII

Inhibition of TK1 activity by selected monoclonal antibodies. To test for inhibition of TK1 activity, the TK assay using ATP was performed using the crude extract of Raji cells described above in regard to isolation of TK1. Replicate assay reactions were prepared. To some reactions, a 20 μl aliquot of supernatant from one of the hybridoma cultures was added, containing between about 0.02 to 0.1 μg of antibody.

The incorporation of radioactivity was compared for control (no antibody) and test reaction to which hybridoma supernatant from selected hybridoma cultures had been added. The data obtained for selected hybridomas are summarized in Table II.

Table II
TK1 Inhibition by Anti-TK1 Antibodies

positive control	56,103 cpm
negative control*	54,327 cpm
Clone # 1	2,012 cpm
Clone # 2	1,557 cpm
Clone # 5	1,743 cpm
Clone # 7	5.067 cpm
Clone # 4	10.338 cpm
Clone # 3	7,252 cpm
Clone # 19	53,109 cpm
* Supernatant from hybridoma cells producing antibodies not binding to TK enzymes	

In the reaction tubes to which were added supernatant samples from clones Nos. 1-5 and 7, the amount of radioactivity incorporated was no more than 20% of the amount of radioactivity in the controls. In contrast, the reaction tube to which supernatant from clone No. 19, one of the fusion products which tested negative by ELISA for TK1 binding, did not inhibit activity of TK1 in the Raji cell extract. It was also found that the ability to inhibit TK1 in this assay was positively correlated with the highest O.D. readings as determined by the ELISA method.

Antibodies from clones nos. 3, 4, 6 and 7 were less efficient in inhibition of TK1 activity in the Raji extract assay (not shown). Therefore, they were not subjected to further screening.

Clones 1, 2 and 5 were again subjected to limiting dilution and colonies derived from this re-cloning procedure were tested once again. The re-cloning procedure was used to ensure that a hybridoma cell line is derived from a single fusion cell and thus produces antibodies which are uniform in isotype and specificity, e.g. monoclonal. Clones Nos. 1, 2 and 5 were placed on deposit under the terms of the Budapest Treaty with the American Type Culture Collection in

Rockville, Maryland U.S.A. on August 11, 1993, and assigned ATCC Nos. HB 11432, HB 11433, and HB 11434, respectively.

Additional screening determined that none of Clones Nos. 1, 2, or 5 produced antibodies binding to TK2 (TK2 antigen produced as described in Example IX, below). However, in a subsequent set of hybridoma clones produced by the procedure of Example V, screening against TK1 antigen as in Example VII and against TK2 antigen as in Example IX, disclosed that at least two clones produced antibodies that were positive for binding to both TK1 and TK2. The latter clones are believed to produce antibodies which will bind with specificity to naturally occurring proteins having 90% sequence homology with a human thymidine kinase (including either TK1 or TK2).

EXAMPLE IX

Isolation of TK2. Isolation of TK2 was performed by FPLC with a Mono-Q column. The starting material was a crude cell extract similar to the Raji extract except that it was prepared from HeLa cells. HeLa is an immortalized human cervical carcinoma cell line available from ATCC under the #CCl-2, which is believed to have high levels of TK2 and very low levels of TK1. When analyzed by non-denaturing PAGE, TK2 also appears to have a monomer form and one or more multimer forms.

As a preliminary step, a crude extract of HeLa cells is run on DEAE-cellulose. Using the CTP assay, the TK2 activity is found to be largely in peaks 18-20, which is quite separate from the region in which TK1 elutes.

Further purification was by FPLC on a Mono Q column. FIG. 5 shows the results of a first run on a Mono Q column of pooled material from peaks 18-20. Buffer A was 0.05 molar Tris-HCl and Buffer B was 0.5 molar Tris-HCl, and the column was run with a gradient of 5% to 45% Buffer B at a flow rate of 1 ml/min. The TK2 activity was assayed in the collected fractions and found to be mostly in fraction 7 (peak 500). Material from peak 500 from several column runs was pooled, concentrated and run a second time with the same buffer gradient and flow rate. FIG. 6 shows the results of the second run. The TK2 activity was found to be in fraction 6 (peak 600). The collected material of fraction 6 is a semi-pure preparation of active TK2 isozyme.

Monoclonal Antibody Specific to TK2

A preparation of TK2 comprising fraction 6 from the second run of the Mono-Q FPLC procedure in Example IX, was used as the antigen. The immunization procedure, harvest of spleen cells and fusion with FO cells was similar to that in Examples V-VI. Screening was performed by ELISA on wells coated with the semi-pure TK2 preparation of Example IX, and the results are given in Table III. The results indicated that antibodies from Clones 2-2, 2-3, 2-4, 2-5, 2-7, 2-8, 2-9, 2-10, 2-12, 2-17 and 2-19 gave the most positive results for binding to the semi-pure TK2. In Table III, the samples designated "2-1A",

Table III

SCREENING OF HYBRIDOMA CLONES FOR ANTI-TK2 ANTIBODIES

Clone	2-1	2-5	2-9	2-13	2-17	2-1A	2-5A	2-9A
O.D. 450	0.460 0.493	0.781 0.796	0.377 0.389	0.361 0.346	0.757 0.707	0.952 0.896	0.973 1.006	0.232 0.204
Clone	2-2	2-6	2-10	2-14	2-18	2-2A	2-6A	2-10A
O.D. 450	0.779 0.781	0.644 0.581	0.316 0.298	0.422 0.395	0.448 0.464	0.951 0.912	0.240 0.250	0.724 0.636
Clone	2-3	2-7	2-11	2-15	2-19	2-3A	2-7A	2-11A
O.D. 450	0.800 0.753	0.660 0.641	0.274 0.237	0.438 0.450	0.825 0.786	0.981 0.997	0.877 0.861	0.197 0.175
Clone	2-4	2-8	2-12	2-16	2-20	2-4A	2-8A	--
O.D. 450	0.731 0.698	0.194 0.214	0.182 0.204	0.447 0.485	0.500 0.600	1.020 1.021	0.714 0.701	0.163 0.199
Control	--	--	--	--	--	--	--	--
O.D. 450	0.079 0.074	0.084 0.073	0.090 0.071	0.083 0.082	0.078 0.076	0.080 0.076	0.076 0.076	0.082 0.078

*Two replicate wells per dilution were analyzed.

"2-5A", etc., represent separate replicate aliquots of antibody from Clones 2-1, 2-5, etc. Clones 2-7, 2-8. 2-9, 2-10, and 2-12 ultimately yielded hybridoma cell lines producing acceptable levels of anti-TK2 antibodies.

For use in tests with patient samples, the selected antibody-producing cell lines were passaged and supernatant was aseptically collected over a period of 3 months. Antibodies were purified by precipitating the supernatants with ammonium sulfate followed either by gel filtration chromatography or by DEAE-cellulose chromatography (diethylaminoethyl cellulose; obtained from Whatman International, Maidstone, Kent, UK under the tradename SEPHADEX). The antibodies were purified by standard methods and conjugated with either HRP-Peroxidase or Alkaline Phosphatase (Bio-Rad). Such procedures are described in ANTIBODIES: A Laboratory Manual by Harlowe and Lane, 1988.

For the determination of serum TK levels in human subjects, fresh samples of peripheral venous blood are collected from the subjects. Serum and mononuclear leukocytes are separated from each sample by conventional methods, and the separated samples are stored frozen until analysis. TK levels in tumor tissues can be determined by preparing an extract of TK from samples of fresh tumor tissue similar to the crude extract of Raji cells of Example I. Preferably, the protein content of the sample is determined so that the amount of TK can be correlated with the amount of total protein in the tumor. An immunoprecipitationassay using the desired anti-TK antibody can then be performed on the extract.

EXAMPLE X

The conjugated antibody from Clone 1 was used to immunostain tumor cells histologically fixed to slides. Diaminobenzidine (DAB) was used as the enzyme substrate in this procedure.

EXAMPLE XI

Detection of active TK1 in serum samples from cancer patients using anti-acTK1 antibody. Serum samples were obtained from cancer patients. Each sample was assayed for TK activity by a method like that of Example I. The same samples were then quantitated blindly on an ELISA test with Clone #1 antibody using different serum dilution levels. A dilution of 1:16,000 was found to give the

best results. The results of the two assays are presented in Table IV. The data were confirmed by Western blot analysis.

In Table IV, the sera are ranked from highest to lowest amount of anti-TK1 bound, by the O.D. reading in the ELISA assay. It can be seen from the TK1 activity measurements, that the correlation between antibody binding data and the standard TK1 activity assay is excellent. The data demonstrate that the anti-AcTK1 antibody can be used to evaluate the serum level of TK1 activity in human subjects. Further, serum from a healthy (non-cancer-bearing) individual bound much less anti-AcTK1 antibody as compared to the lowest-ranked serum of a cancer patient. Thus, the anti-AcTK1 antibody is useful to distinguish between

Table IV

Comparison of TK1 Activity Measured by ^{3}H-thymidine Incorporation and Anti-AcTK1 Binding

Serum #	TK1 assay	Rank*	O.D.450 nm
1	2498	5	.443
2	2376	5	.430
3	8251	2	.865
4	6254	3	.728
5	2214	5	.420
6	11477	1	1.542
7	2509	5	.450
8	4785	4	.592
0[1]	-	8	.250

The O.D. 450 readings represent the amount of bound Clone #1 antibody measured by ELISA. The TK activity values are the cpm of ^{3}H incorporated per minute.
* determined from the ELISA data
[1] Healthy (non-cancer) control

serum of cancer-bearing individuals and serum from healthy non-cancerous individuals.

It has been further found that the levels of TK activity, and particularly of TK1 activity, can be used as a reliable predictor of the likelihood of tumor recurrence in breast cancer patients. Table V summarizes the results of a study of TK activity in samples from untreated primary tumors that were surgically removed from 86 patients, 13 of which later experienced a recurrence of disease.

In this study, it was also found by comparing TK levels with estrogen receptor status, that the TK level accurately predicts which patients in the estrogen receptor positive group later showed recurrence and which in the estrogen receptor negative group did not show recurrence. Of 57 estrogen receptor positive patients (patients whose tumors had high numbers of estrogen receptors), 4 had recurrence of disease. The average TK activity level measured in the tumors from those four patients was 289,717 cpm/min, as compared with 161,674 cpm/min for tumors from patients who had no recurrence. Also, the percentage of the activity attributable to TK2 was about 76% in the no-recurrence group, vs. 41% in the four who had recurrence.

Conversely, of 29 estrogen receptor negative patients (those whose tumors had few or no estrogen receptors), the 20 who did not have

TABLE V

Tumor TK levels and recurrence

Patients	Number	Total Tumor TK*	Statistical Significance	%TK2	Statistical Significance
No Recurrence	73	144961.9	p<0.001	74.5	p<0.001
Recurrence	13	351693.5		41.7	

*cpm/min of reaction time per mg protein

recurrence had an average TK activity level of 100675 cpm/min with about 71% being TK2 activity, as compared to 379238 cpm/min with about 42% being TK2 activity for the group that did have recurrence. Thus, the level of TK activity, and the relative proportions of TK2 to TK1 activity, were both better predictors of recurrence than estrogen receptor status.

With respect to leukemia patients, it has been found that the level of TK1 activity in serum can be used to detect relapse, often before any other symptom is evident.

The anti-AcTK1 antibody is useful to screen serum and tissue from cancer patients both for study purposes and for use in diagnosis and treatment of tumor patients. The anti-AcTK1 antibody, the anti-totalTK1 antibody, and/or the anit-TK2 antibody, may also be useful for serum screening in certain other kinds of blood disorders, such as pernicious anemia, where TK activity has been linked to disease status. The antibodies may further be useful for testing of patients with certain viral diseases including morbilli, rubella and herpes, where it has been found that the levels of thymidine kinase are elevated during the acute phase of the disease.

Because the anti-AcTK1 antibody detects only active-form TK1, it can be used in place of the radioactive thymidine incorporation assay to evaluate activity. The use of the antibody for evaluating TK1 activity in tumors and serum of cancer patients will make this test practical for use on a wide scale, whereas the activity assay is generally more difficult to perform.

Also, the antibody may be useful for targeted tumor therapy. That is, an anti-tumor agent may be coupled to the antibody, which will selectively bind to tumor cells expressing large amounts of TK1. In this manner the anti-tumor agent is targeted to tumor cells and the killing of tumor cells is enhanced versus killing of normal cells.

The invention may also be embodied as a hybridoma cell line obtained by injecting a host animal with a TK preparation, isolating activated B cells from the spleen of the injected animal, fusing the activated B cells with myeloma cells, and individually screening clones of the fused cells to select a hybridoma cell line which produces anti-TK antibodies having desired binding specificities. In a highly preferred embodiment, the TK preparation is derived from Raji cells. In another preferred embodiment, the TK preparation is a substantially homogeneous preparation of tetramer and/or active TK. Antibodies produced by a hybridoma obtained by one of the methods described in this application are also encompassed by the invention.

Still further, the invention encompasses methods of using anti-TK antibodies, and a kit for performing the methods. A method of determining the serum level of a thymidine kinase enzyme comprises the following steps: obtaining a serum sample from a patient, providing a monoclonal antibody which selectively binds to thymidine kinase enzyme, contacting the monoclonal antibody with the sample, and determining the amount of monoclonal antibody bound to thymidine kinase protein in the sample. The thymidine kinase enzyme or enzyme forms which may be measured by this method using a single monoclonal antibody including the following categories: total TK1 including monomeric and multimeric forms and active and inactive forms; active form TK1 only; TK2 only.

An alternate embodiment is a method for evaluating the level of thymidine kinase in a solid tumor sample. This method is essentially the same as that for evaluating serum thymidine kinase levels, except that the step of providing a serum sample is replaced by a step of providing a tumor sample. The tumor sample may be a fresh or frozen tissue sample or a histological slide preparation.

A kit for performing the above methods may comprise one or more monoclonal antibodies selected from the group including anti-total-TK1, anti-AcTK1, and anti-TK2. In one embodiment, the monoclonal antibody is conjugated to an enzyme useful in an ELISA assay or to another detectable marker such as a fluorescent dye, radioactive isotope, or the like. Alternatively, the kit may further include an anti-mouse antibody which is enzyme-conjugated for detection by ELISA or otherwise labelled.

A method of predicting the likelihood of recurrence of a solid tumor in a patient at initial diagnosis comprises the steps of establishing a normal range for tissue TK activity, obtaining a sample of a primary tumor from a patient, determining the amount of TK enzyme in the patient sample to produce a patient TK value, and comparing the patient TK value it to a normal value; and if it exceeds the normal range by a significant amount, predicting that the tumor is likely to recur, and if it does not significantly exceed the normal range, predicting that recurrence is unlikely. The step of establishing a normal range may be performed in several ways. In one embodiment, one or more samples of the patient's own normal tissue may be used for comparison. In another embodiment,

a normal range may be established from a study of normal tissues in healthy (no disease) individuals, or from patients known to be in remission.

In a highly preferred embodiment, the TK measurement is selectively of TK1 activity or provides a comparison of TK1 and TK2 activity levels. In a further preferred embodiment, the level of active TK1 is measured using an anti-AcTK1 antibody.

In an alternate preferred embodiment, the total TK1 content is measured using an anti-total-TK1 antibody. Still another embodiment of the methods employs an anti-total-TK1 antibody and an anti-TK2 antibody. In bladder cancer, a preferred alternate embodiment employs an anti-TK2 antibody.

A method of determining whether disease has recurred in a patient being treated for a cancer or blood disorder, including breast cancer, leukemia and lymphoid cancer, comprises the steps of taking a series of samples of the serum of a cancer patient at regular intervals, measuring the amount of TK in the samples, comparing the amount of TK among the samples, and when the amount of TK in later samples exceeds the amount of earlier samples by a significant degree, determining that the disease is recurring.

In a preferred embodiment, the measurements are of TK1 activity in the sample as determined using an anti-active TK1 monoclonal antibody. An alternate embodiment employs an anti-total-TK1 antibody. Still other embodiments utilize an anti-total TK (both TK1 and TK2) antibody, or comparative measurements of TK isozymes using two or more of the noted antibodies.

To summarize, the present application contains at least the following teachings: that only TK1 tetramers or multimers have significant activity; of convenient sources (Raji cells, HeLa cells) and methods for isolation of large quantities of TK1, of active TK1, and of TK2 for use as antigen and in screening to select desired hybridomas; and of screening procedures to identify antibodies having different specificities. It will be apparent that, given these and other teachings of this application, one of ordinary skill could obtain the hybridomas and antibodies described and claimed herein, as well as monoclonal antibodies having specificity for any desired category of thymidine kinase isozymes.

The Claims

What is claimed is:

1. A monoclonal antibody which binds with specificity to all forms of thymidine kinase protein present in a sample, including TK2, active and inactive forms of TK1, and monomer and multimer forms of TK1.

2. A monoclonal antibody which binds with specificity to tetramer TK1.

3. The monoclonal antibody of Claim 2, which is further specific for enzymatically active TK1 isozyme.

4. The monoclonal antibody of any of Claims 1-3, which inhibits the phosphorylation of thymidine by TK1 in an enzymatic assay.

5. A monoclonal antibody which binds with specificity to TK2.

6. A hybridoma producing a monoclonal antibody according to any of Claims 1-5.

7. A kit containing two or more monoclonal antibodies mutually configured and selected to determine the relative amounts of TK1 and TK2 isozymes in a sample, said kit comprising a first antibody which binds with specificity to TK1 and a second antibody which binds with specificity to total TK defined as protein having 90% or greater sequence homology to either TK1 or TK2.

8. The kit of Claim 7, wherein said first antibody is selected from the group consisting of: antibody specific for active-form TK1, antibody specific for tetramer TK1, and antibody specific for all forms of TK1 including monomer, multimer, tetramer and active and inactive forms.

9. The kit of Claim 7, in which said first antibody is an antibody which binds with specificity to active-form TK1.

10. A method of determining the level of thymidine kinase in a clinical sample, comprising the steps of:
providing a monoclonal antibody according to any of Claims 1-5;
contacting the monoclonal antibody with a clinical sample; and
determining the amount of monoclonal antibody binding in the sample.

11. A method of determining whether relapse is occurring in a subject who has been treated for cancer and whose cancerous disease is in remission, comprising the steps of:
taking a series of samples of the serum of the subject at regular intervals following the onset of the remission;
measuring the amount of thymidine kinase in the samples;
comparing the amount of thymidine kinase in later samples in the series with the amount in earlier samples in the series, and when the amount of thymidine kinase in the later samples exceeds the amount in the earlier samples by a significant degree, determining that relapse is occurring.

12. The method of Claim 11, wherein said step of measuring the amount of thymidine kinase comprises the steps of:
providing a tagged anti-thymidine kinase monoclonal antibody according to any of Claims 1-5, prior to obtaining the series of samples; and
determining the respective amounts of said tagged anti-thymidine kinase monoclonal antibody which bind to protein in healthy subjects and in subjects having active cancerous disease.

13. The method of Claim 12, further including the steps of: determining the relative proportions of TK1 and TK2 activity in the samples, and upon observing a significant increase in the amount of TK1 activity, determining that relapse is occurring.

14. A method of predicting the likelihood of recurrence of a tumor in a patient at the time of initial diagnosis, comprising the steps of:
establishing a normal range for tissue thymidine kinase activity from a series of samples from healthy subjects;
obtaining a sample of a primary tumor from a tumorous subject;
determining the amount of thymidine kinase enzyme in the tumor sample to produce a tumor TK value; and
comparing the tumor TK value it to the normal value, and if it significantly exceeds the normal range, predicting that the tumor is likely to recur, and if the tumor TK value does not significantly exceed the normal range, predicting that recurrence is unlikely.

15. The method of Claim 14, wherein said step of determining the amount of thymidine kinase enzyme comprises the steps of:
providing a tagged anti-thymidine kinase monoclonal antibody according to any of Claims 1-5, prior to establishing the normal range and obtaining the tumor sample; and
determining the respective amounts of said tagged anti-thymidine kinase monoclonal antibody which bind to protein in the samples from healthy subjects and in a series of tumor samples of tumors of tumorous subjects.

16. A method of making a hybridoma cell line which produces a monoclonal antibody against thymidine kinase, comprising the steps of:
providing a thymidine kinase preparation;
injecting a host animal with the thymidine kinase preparation, and waiting a sufficient time for the animal to mount an immune response against the antigen preparation;
removing activated B lymphocytes from the injected animal;
fusing the activated B lymphocytes with myeloma cells from a cultured myeloma cell line to produce a plurality of hybridoma cells, and individually culturing selected ones of the hybridoma cells to produce a plurality of hybridoma clones; and

screening said hybridoma cultures to select a hybridoma clone which secretes an antibody that binds with specificity to a thymidine kinase enzyme.

17. The method of Claim 16, wherein said thymidine kinase preparation is a substantially homogeneous preparation of active TK or multimer TK.

18. The method of Claim 16, wherein said thymidine kinase preparation is prepared from Raji cells.

19. A hybridoma cell line obtainable by the method of any of Claims 16, 17, or 18.

20. An antibody obtainable by culturing a hybridoma cell line obtained by the method of any of Claims 16, 17, or 18.

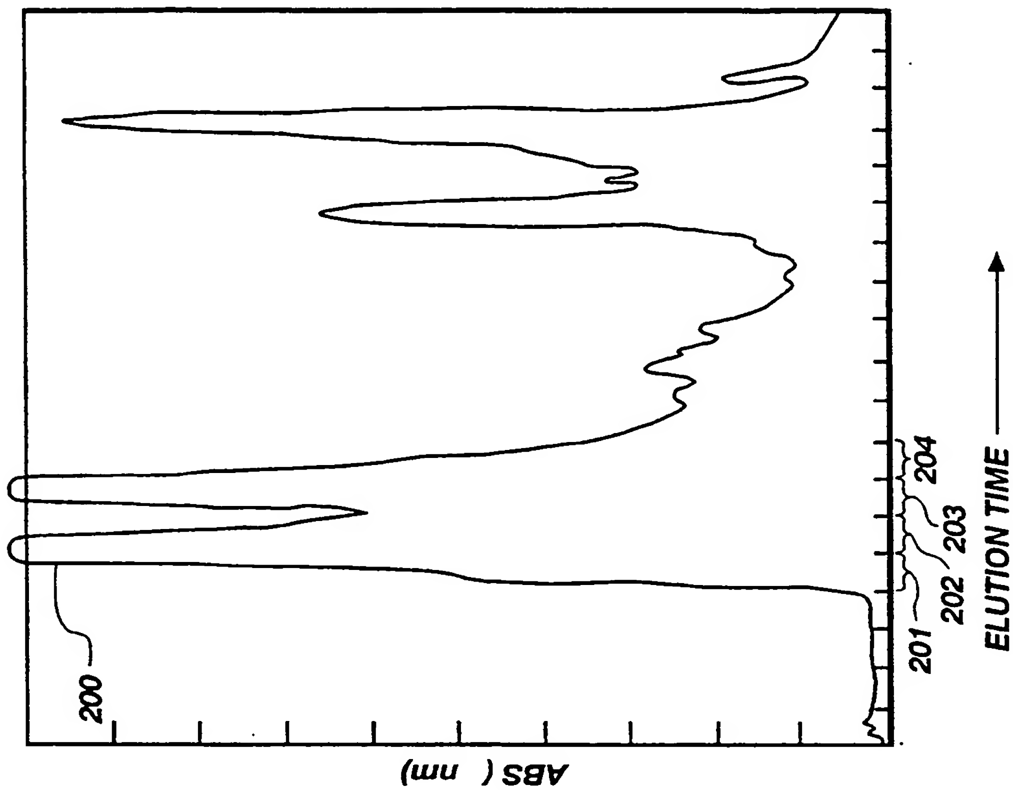
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Fig. 2

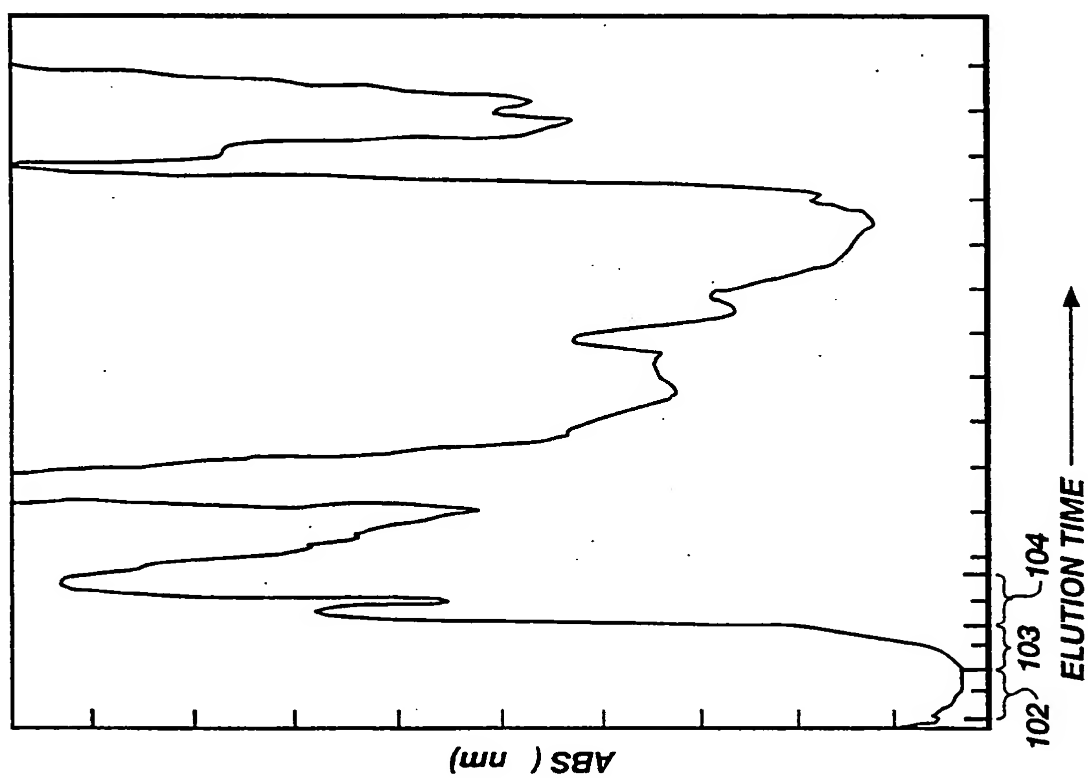
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Fig. 1

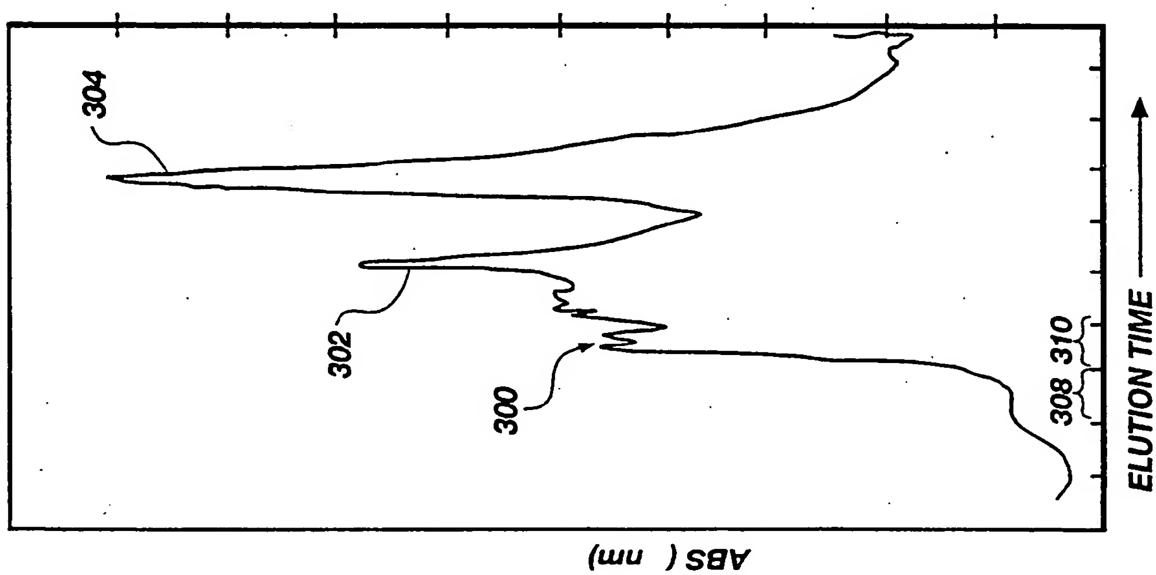

Fig. 3

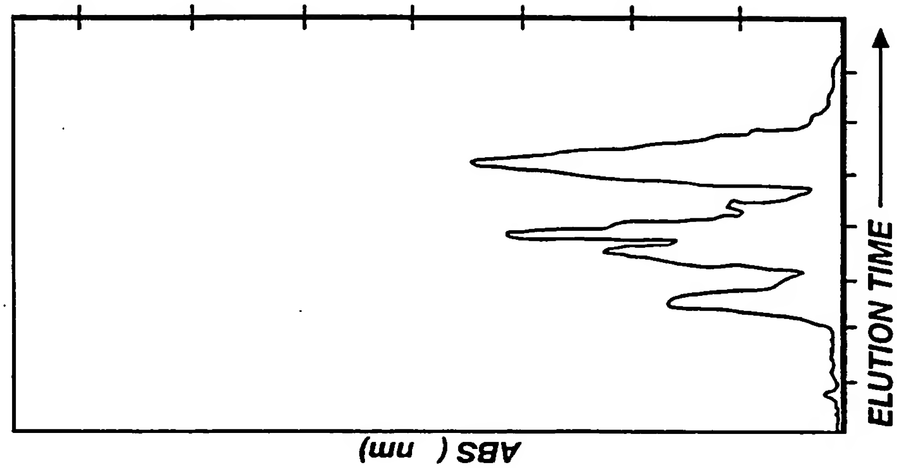

Fig. 4

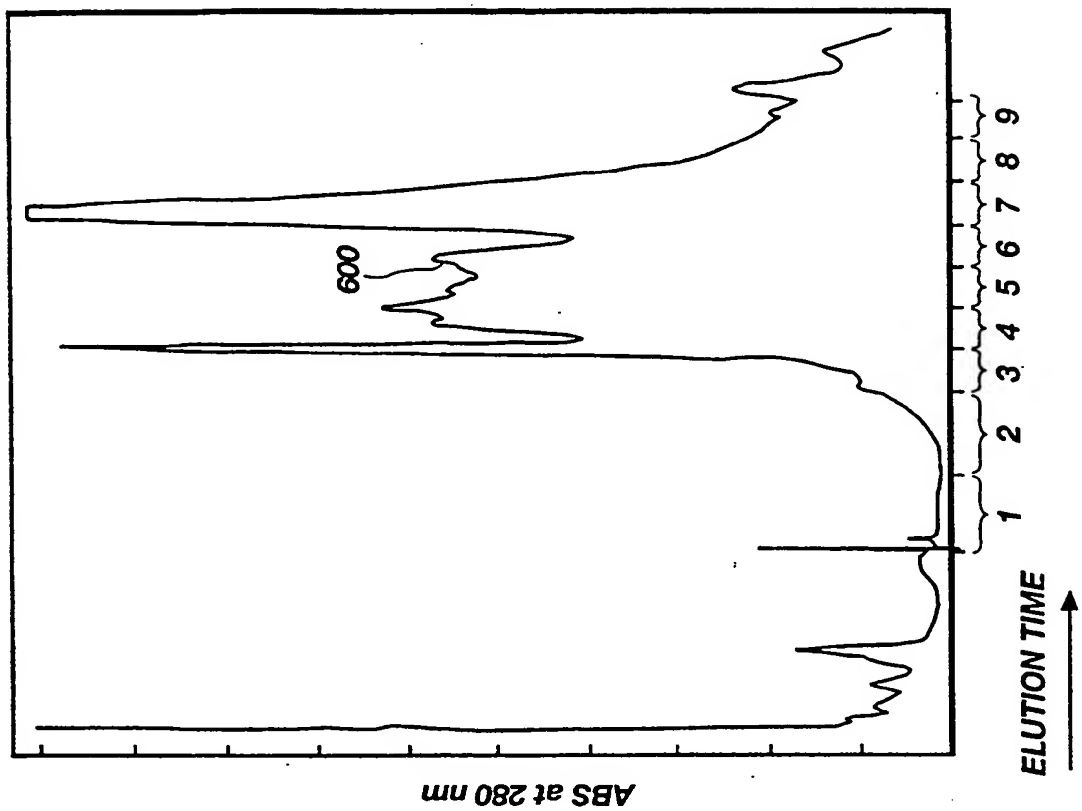
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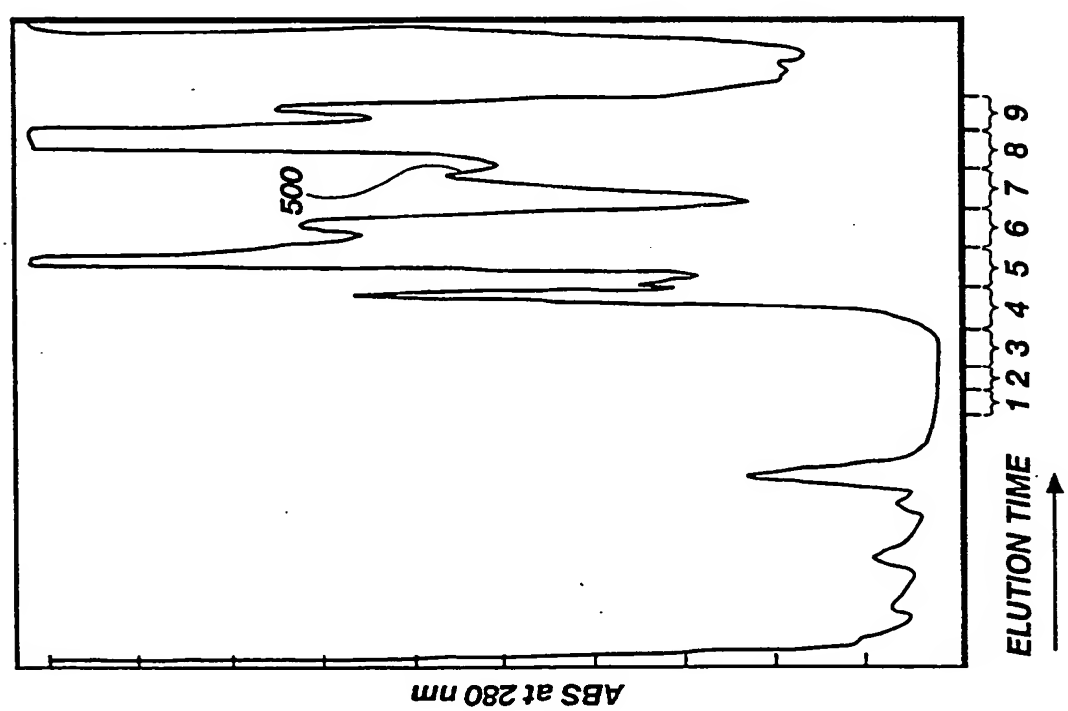
Fig. 6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ABS at 280 nm

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09022

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 16/40; C12N 5/16, 5/18, 15/02; C12P 21/08; C12Q 1/25; G01N 33/53
US CL : 530/388.26; 435/7.1, 7.4, 7.92, 70.21, 172.2, 240.27, 810
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.26; 435/7.1, 7.4, 7.92, 70.21, 172.2, 240.27, 810

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,317,877 (BALIS ET AL.) 02 March 1982, col. 1, lines 11-14 and col. 4, lines 46-52.	1-10, 16-20
Y	EP, A, 0,255,431 (JOUAN ET AL.) 02 March 1988, see claim 12 in particular.	1-10, 16-20
Y	Gene, Volume 52, issued 1987, E. Flemington et al, "Sequence, Structure and Promoter Characterization of the Human Thymidine Kinase Gene", pages 267-277, especially page 275.	1-10, 16-20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 OCTOBER 1994	25 NOV 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	CHRISTINA CHAN H. Vuyza for
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical Pharmacology, Volume 31, No. 6, issued 1982, J. Balzarini et al, "Role of Thymidine Kinase in the Inhibitory Activity of 5-Substituted-2'-Deoxyuridines on the Growth of Human and Murine Tumor Cell Lines", pages 1089-1095, especially page 1089.	1-10, 16-20
Y	Eur. J. Biochem., Volume 206, No. 2, issued June 1992, O. Jansson et al, "Mammalian Thymidine Kinase 2, Direct Photoaffinity Labeling with [^{32}P]dTTP of the Enzyme from Spleen, Liver, heart and brain", pages 485-490, especially page 485.	1-10, 16-20
Y	Proc. Natl. Acad. Sci. USA, Volume 80, issued September 1983, H. D. Bradshaw, "Molecular Cloning and Cell Cycle-Specific Regulation of a Functional Human Thymidine Kinase Gene", pages 5588-5591, especially page 5588.	1-10, 16-20
Y	Journal of Immunological Methods, Volume 39, issued 1980, J. W. Goding, "Antibody Production by Hybridomas", pages 285-309, see the entire document.	1-10, 16-20

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-10 and 16-20

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-10 and 16-20, drawn to a monoclonal antibody, a hybridoma, a kit, a method of determining the level of thymidine kinase in a clinical sample and a method of making a hybridoma cell line, Classes 530 and 435, Subclasses 388.26 and 7.1+, respectively.

II. Claims 11-13, drawn to a method of determining whether relapse is occurring in a subject , Classes 435 and 436, Subclasses 7.23+ and 64+, respectively.

III. Claims 14-15, drawn to a method of predicting the likelihood of recurrence of a tumor in a patient. Classes 435 and 436, Subclasses 7.23+ and 64+, respectively.

The inventions as grouped are distinct, each from the other, because they represent different inventive endeavors. The monoclonal antibody, the hybridoma, the kit, the method of determining the level of thymidine kinase in a clinical sample and the method of making a hybridoma cell line in Group I would not suggest the method in Group II or the method in Group III. Groups I-III are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.